

CULTURE-GROWN SPORANGIA OF *PHYTOPHTHORA INFESTANS* DIFFER FROM
SPORANGIA HARVESTED FROM LIVING PLANT TISSUE

A Thesis

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Master of Science

by

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ABSTRACT

To assess the impact growth substrate has on the resulting sporangia, field and laboratory based inoculation experiments were carried out using three isolates of *P. infestans* clonal lineage US-23. Isolates were grown in pure culture on agar plates as well as on detached *Solanum lycopersicum* leaflets in moist chambers. Sporangia harvested from each of these culture types and for each isolate was used as inoculum in two independent field trials and one laboratory based trial in the summer of 2015. Disease assessment at 144 hours after inoculation was very consistent across all trials. In every case, culture derived sporangia produced significantly less disease than sporangia of the same isolate washed from sporulating leaflets. Genes associated with necrotrophy and host cell death were up regulated in culture grown sporangia, while genes associated with biotrophic growth and disease were more up regulated in sporangia from leaflets.

BIOGRAPHICAL SKETCH

Sean Patev began his college career at the University of Massachusetts Amherst in 2008. Initially studying chemistry, he soon found himself far more interested in the world of microbes. After changing majors to Microbiology and working in the Griffith lab on *Bacillus subtilis*, Sean found himself looking for a real-world connection for microbiology. He found this applied microbiology in the field of Plant Pathology, introduced to him in Dr. Robert Wick's introductory plant pathology course. Sean was immediately hooked, and not known to take new interests in moderation he proceeded to enroll in every available plant pathology course at UMass. Through a combination of work study, independent research credit, and volunteer time, Sean spent the last three years of his time at UMass continually employed in plant pathology work. For one year he worked in the Cooley lab under Arthur Tuttle, assisting in field work concerning apple scab, sooty blotch, fly spec, plum curculio, and other IPM projects. Simultaneously, and up until his departure from Amherst, Sean also worked in the Wick lab. There he studied a wide variety of plant diseases, focusing primarily on downy mildew of basil, but reaching into nematology, sudden oak death, turf grass diseases, and a wide variety of diagnostic work for the Massachusetts Plant Disease Diagnostic Clinic. It was during this time that late blight of potato and tomato reemerged as a serious problem in the United States. The significant sampling and culturing effort of the Wick lab introduced this pathogen to Sean, and it eventually became the focus of his Masters research. At the urging of Dr. Wick, Sean applied to Cornell University for graduate study, focusing on the pathogen he had spent so much time collecting in years before, *Phytophthora infestans*. He studied this pathogen under Dr. William Fry, culminating in the Master's thesis presented here.

To my family, friends, and mentors
whose patience and support were truly invaluable.

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TABLE OF CONTENTS

Biographical Sketch.....	iii
Dedication.....	iv
Acknowledgements.....	v
Table of Contents.....	vi
List of Figures.....	vii
List of Tables.....	viii
Chapter 1: Culture-grown sporangia of <i>Phytophthora infestans</i> differ from sporangia harvested from living plant tissue.....	1
References.....	16

LIST OF FIGURES

Figure 1: Arachidonic acid treatment on potato leaflets.....	2
Figure 2: Disease at 144hai of field inoculated tomatoes.....	11
Figure 3: Disease at 156hai of laboratory inoculated tomatoes (NV and 112).....	12
Figure 4: Disease at 144hai of laboratory inoculated tomatoes (GC and NV).....	14

LIST OF TABLES

Table 1: Treatment types.....	4
Table 2: Primers.....	9
Table 3: Field trial disease severity.....	10
Table 4: Lab trial disease severity (NV and 112)	12
Table 5: INF-1 and RxLR expression.....	13
Table 6: Lab trial disease severity (GC, NV, and 112).....	13
Table 7: Gene expression compared to average disease.....	15

Culture-grown sporangia of *Phytophthora infestans* differ from sporangia harvested from living
plant tissue

Phytophthora infestans is a devastating pathogen of tomato and potato, known worldwide for its impacts on agriculture. The disease it causes, late blight, is among the most widely known plant diseases for such significant events as the Irish potato famine of the 1840s.¹ More recently, this pathogen caused widespread crop destruction and significant monetary loss in the late blight pandemic of 2009.² Despite the availability of effective fungicides, control is still an issue and significant amounts of fungicide are used. It has been estimated that over \$6 billion is lost each year from crop damage and chemical control on potato alone.³ As a result, *P. infestans* is as relevant a pathogen today as it has ever been, and is the topic of some 3,000 publications each year.⁴ For this reason, developing new techniques and re-visiting established ones is an important part of understanding the pathosystem.⁵ This thesis assesses one of the most basic aspects of investigating *P. infestans*, in an attempt to further improve research on this subject.

For much research concerning *Phytophthora infestans*, inoculation of plant material is essential. Consideration is regularly given to pathogen genotype, sporangium concentration, release of zoospores, and method of application.^{6,7} The way that sporangia are produced varies widely among researchers and is sometimes not described in publication.⁸ The two major methods for producing sporangia are from leaf lesions as described by Porter et al. (2004)⁶, or from monoxenic cultures growing on any of a diverse number of media.^{7,9–11} There is also a wide diversity of sporangium concentrations used in inoculations. For example, in Zuluaga et al. (2015)¹² sporangia harvested from tomato leaflets were applied at a rate of 4,000 sporangia per

ml. Similarly, other experiments using leaflet-derived sporangia used concentrations as low as 300 sporangia/ml¹³. In contrast, a suspension of 150,000 sporangia/ml harvested from pure *P. infestans* culture on rye agar was used in 2009 by Vargas et al.⁹

There are some indications that sporangia from lesions on leaflets have different characteristics than sporangia produced in pure culture on sterile media. Mizubuti et al (2000) reported that sporangia from culture were dramatically more sensitive to solar radiation than were sporangia produced from lesions on leaflets.¹⁴ Differences appeared in another experiment investigating arachidonic acid as a “priming” agent for up-regulating defense genes in tomato.¹⁵ Investigations from our lab found that when plants treated with arachidonic acid were inoculated with sporangia produced in pure culture, they appeared resistant; however, when they were inoculated with sporangia produced on leaflet lesions, they were not resistant (Figure 1).⁵ It seems reasonable to infer then, that the substrate on which sporangia are produced may have significant effects on the behavior of those sporangia and that subsequent disease may also be influenced

The goal of this study was to test the hypothesis that sporangia produced in pure culture are less aggressive than those produced on leaflet lesions, and if differences in aggressiveness were detected, to characterize some of the differences during infection. I used three isolates of the US-23 clonal lineage of *P. infestans* and recorded disease severity in susceptible tomato plants when the plants were inoculated in the lab or in the field with sporangia obtained from pure

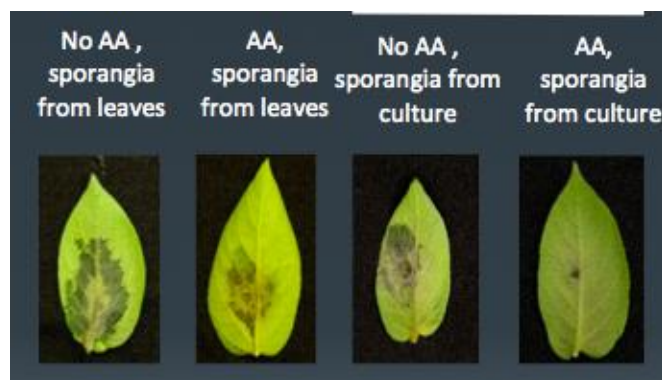


Figure 1. Potato leaflets treated or not with arachidonic acid (AA) and inoculated with sporangia from culture or from leaves (living tissue). From Fry 2016 ⁵

culture or from leaflet lesions in moist chambers. During these experiments, it became clear that there might be differences in gene expression of sporangia from different sources even before infection. Thus, an additional goal was to test the hypothesis that gene expression in sporangia produced in pure culture is different from that in sporangia produced from lesions on leaflets. I studied the dynamics of select gene expression in *P. infestans* sporangia harvested from different substrates, and collected tissue throughout disease progression for future analysis with RNA sequencing.

MATERIALS

Isolates

Each of the three isolates of the US-23 lineage had been obtained from the field less than two years before analysis. Isolate “140112”, referred to as “112” from here on, was cultured from diseased commercial tomato plants gathered in New York State in 2014. After identifying the isolate as clonal lineage US-23 with microsatellites,¹⁶ it was kept in culture on agar plates containing a mixture of ½ pea¹⁷ and ½ rye B agar.¹⁸ To move an isolate to live tissue from pure culture, sporangia were washed from the pure culture and drops (each 200µl) of this suspension were deposited on the underside of tomato leaflets in moist chambers (inverted water agar dishes).¹² Subsequent transfers from leaflet to leaflet were carried out by touching healthy leaflets to diseased sporulating ones and placing the inoculated leaflets in a new water agar moist chamber. Agar cultures were transferred by cutting and moving colonized agar cubes from the old culture to a fresh pea/rye agar plate. Agar and tissue cultures were kept at 20°C, approximate room temperature in the lab, on the benchtop.

Isolate “Newark Valley” or “NV” was cultured from diseased tomato plants in New York State in the early summer of 2015, shortly before the field experiments (see below) were initiated. After identifying the isolate as US-23 by microsatellite analysis,¹⁶ the isolate was also cultured on both pea/rye agar plates and detached tomato leaflets in moist chambers. Touch transfers as described above were used to inoculate healthy leaflets and maintain the isolate on plant material. Agar culture transfers were carried out by transferring cubes of colonized agar as described previously. After use in inoculation experiments in 2015 this isolate was maintained on pea/rye agar as described above until it was used again in 2016.

Isolate “GCREC” or “GC” was cultured from diseased tomato plants submitted from Florida to our lab for analysis in spring 2016. It was identified as US-23 by microsatellite analysis and cultured on pea/rye agar and detached leaflets, using the same techniques described for establishing the other isolates in culture. This isolate was not utilized in the 2015 inoculation studies, and was therefore not kept in long term culture before use in 2016.

Field Experiments

There were two field trials of the same experiment, one consisting of two randomized complete blocks and the other consisting of three randomized complete blocks. The susceptible tomato cultivar ‘Jet Star’ was used in both trials. Plants were grown in the greenhouse until they were about 5 inches in height. These plants were hardened off in cold frames for one week and then transplanted to the field at the Thompson Research Farm in Freeville NY. The first trial was transplanted on July 10 and the second trial was transplanted on 16 July 2015. There were

Water
112P
112L
NVP
NVL

Table 1: Treatment types. The suffix "P" represents sporangia taken from pure agar culture, and "L" signifies sporangia taken from sporulating tomato leaflets.

five treatments (three-plant plots) in each block. Fallow soil surrounded each plot for a distance of 9 ft. Treatments consisted of the following inoculations: 5ml water (mock inoculation) or a sporangial suspension (5,000 sporangia/ml) from pure culture or from leaf lesions of 112 or NV. Each of these five inoculations was applied to two plots within a block (Table1). Inoculated tissues were harvested at 12 or 60 hours after inoculation (hai) for later RNA sequencing and analysis. Inoculation of the first trial occurred on 11 August, and inoculation of the second trial occurred on 14 August.

Moist Chamber Experiments

The treatments applied in the field experiment (see above) were also applied to detached leaflets in experiments conducted in moist chambers in the lab. Leaflets were obtained from tomato plants (cultivar JetStar) growing in the greenhouse. The leaflets were detached from fully developed upper leaves of plants 6-8 weeks from seeding. The moist chambers consisted of 15 cm Petri plates containing 20 ml of 0.05% water agar. The plates were inverted (water agar on top in the smaller half) and four-five tomato leaflets were placed in the bottom (larger half). There were three independent trials of moist chamber experiments with one moist chamber per treatment in each trial. When tissue was harvested for analysis (12 or 60 hai) one or two leaflets were retained in the moist chamber and the severity of disease (see below) on these leaflets was assessed at 144 hai. Sporangia for each trial of the moist chamber experiment were obtained as described below. The leaflets were spray inoculated at a concentration of 5,000 sporangia per mL, using 1.5 mL per moist chamber. After inoculation, all moist chambers were kept at 20°C on the laboratory bench top.

Inoculum

Sporangia were obtained from pure culture or from leaf lesions. Sporangia from pure cultures were harvested when the isolate had covered about 70 % of the surface of the pea/rye agar (typically 10-12 days after starting the culture). Sporangia were washed off with distilled water and concentration in this initial suspension was determined using a hemacytometer, then adjusted to 5000/ml. Subsequently, the suspension was placed at 5°C for 30 minutes to promote zoospore release and then re-assessed on the hemacytometer to confirm concentration and germination. Sporangial suspensions from leaflet lesions were prepared by washing two tomato leaflets (on which sporulation occurred on approximately 90% of the leaf surface) in distilled water. The resulting suspension was then adjusted to 5000/ml and incubated at 5°C as described above. For each trial of each experiment, all inoculations for all treatments occurred on the same day and all sporangial suspensions were prepared on the day of inoculation.

For the first field experiment, the NV-L cultures had been transferred three times onto leaflets since isolation, and cultures on pea/rye agar (NV-P) had been transferred twice on pea/rye agar. For the first field experiment, leaflet cultures of isolate 112 had been passed through leaflets three times. For the second field experiment, each isolate had been transferred an additional time on its appropriate substrate.

The first moist chamber experiments occurred after the field experiments, so the isolates had been on their respective media for more passages. The three trials of this moist chamber experiment occurred sequentially so each isolate had an increasing number of passages on its respective medium from trial one to trial three of the moist chamber experiment. All isolates had the same number of passages on their respective substrates within each trial.

Inoculation

Water or sporangial suspensions were applied to plant surfaces using Preval 100ml aerosol sprayers. In the field, 5ml of suspension was applied per three-plant plot on the evening of 11 August 2015 for the first field experiment and on the evening of 14 August 2015 for the second. Inoculations were conducted just before sundown to plants that had been wetted via 30 minutes of sprinkler irrigation to provide optimal infection conditions. The volume of 5ml was sufficient to thoroughly cover the small plants without significant excess. In the first moist chamber experiment, leaves in moist chambers were sprayed with 1ml of the appropriate sporangial suspension or distilled water. There were three replications of this experiment, and inoculation occurred on the evenings of 17 August, 8 September, and 22 September 2015. Chambers were sealed with Parafilm to maintain humidity and promote good infection conditions.

Tissue Harvesting

In both field trial experiments, leaflets were flash-frozen in liquid Nitrogen immediately after harvesting followed by storage in 50ml tubes at -80°C. Sampling occurred at 12 or 60 hours after inoculation. After sampling in the field experiment, one plant of each treatment in each replication was retained for future disease assessment; all remaining plants were uprooted and bagged for disposal.

In the moist chamber experiments, leaflets were harvested at 12 or 60hai and again flash-frozen in liquid nitrogen, followed by storage at -80°C. Unsampled leaflets were retained in the resealed chamber until assessment at 144hai.

Disease Assessment

In all experiments the retained tissue was photographed and also assessed visually. Each of the retained plants in the field experiments was uprooted and placed into a plastic bag at 84 hai and kept at 21 °C until observation at 144 hai. Bagging plants at 84hai prevented sporulation in the field, minimizing the risk of contaminating other experiments or serving as an inoculum source for neighboring commercial growers. At 144 hai, plants were removed from their bags, photographed and assessed for disease severity using the key from James, C (1971) which standardizes disease measurement as percent coverage of foliar surfaces in sporulating lesions.¹⁹ In the moist chamber experiment, two leaflets in each treatment were retained for observation at 144hai. These leaflets were assessed using the key from James, C (1971)¹⁹ and were subsequently photographed at 156 hai.

Gene Expression in Sporangia

To investigate gene expression, samples of pure sporangial suspension were collected and concentrated. Pure sporangial suspensions of GC, NV, and 112 were prepared as described for inoculum preparation except that these suspensions were concentrated by centrifugation (4 min at 14,000 rpm) in 2ml snap-top tubes to produce pellets of sporangia. After decanting the supernatant, the pellets were frozen in liquid Nitrogen, and then stored at -80C until further processing (See RNA extraction below).

Total RNA Extraction

Total RNA from all tissue and sporangial samples was extracted for subsequent processing into cDNA libraries for sequencing or reverse transcription for real-time qPCR. Frozen leaflets from each sampling event of laboratory and field trials were ground using a

mortar and pestle in liquid Nitrogen. RNA was extracted from 100mg of homogenized leaf tissue using a Qiagen RNEasy Mini kit.

Concentrated sporangial suspensions were ground in extraction buffer (Lexogen Split RNA “IB”) using ceramic bead homogenization tubes in a bead beater, and RNA was extracted using the Lexogen Split RNA kit. Extractions resulting in more than 100ng RNA/ul with absorption characteristics of 260/280 above 2.0 and 260/230 above 1.8 as assessed on a Thermo Scientific NanoDrop were frozen at -80°C for future use.

Real-time PCR

Real-time qPCR was conducted to assess potential differential expression of a selected set of genes in *P. infestans* sporangia. Total RNA extracted was transcribed to cDNA using the Applied Biosystems cDNA synthesis kit for reverse transcription. Real-time PCR was carried out on an AbiPrism 7000 and calculation of relative expression was carried out following the protocols outlined in by Pfaffl²⁰ *Phytophthora* genes of interest included an RxLR effector (PITG_16705.2), elicitor INF-1 (PITG_12551), and actin (PITG_14461) as a normalizer. Primers used to amplify regions of these targeted genes are listed in Table 2.

Target Gene	Forward Primer	Reverse Primer
RxLR	5'-CACAAGTGGCTGGCATATCT-3'	5'-GCCGAACGGTTGGTAACTAATA-3'
INF-1	5'-GTGGCGCTCGTAAGCATCCT-3'	5'-CATAGCGACGCACACGTAGA-3'
Actin	5'-AGCTGTGGGCACACTTAAT-3'	5'-CTGGTCCATGCTCTCATTCA-3'

Table 2. Forward and Reverse primers for amplification of selected *P. infestans* gene regions.

cDNA Library Preparation for RNA Sequencing

For later RNA sequencing, cDNA libraries for Illumina sequencing were synthesized from previously prepared total RNA using the Lexogen SENSE mRNA library kit. This kit excluded ribosomal RNA using magnetic beads which associated with poly-A tails found only

on mRNA. This allowed ribosomal RNA to be washed away, maximizing coverage of expressed genes when the resulting libraries are sequenced. Real-time PCR was used to establish accurate cycling for barcode incorporation and final library amplification, avoiding over or under cycling libraries which could skew transcript counts during sequencing. Library fragment size was assessed by fragment analysis, with concentration data from Qubit fluometric analysis of each library. A 2nM equimolar pool was prepared for each of two lanes of Illumina HiSeq 4000. Lane one contained 12 and 60 hour time points from field trial 1 and two moist chamber replicates, while lane 2 contained field trial 2, one moist chamber replicate, and cDNA from RNA extracts of sporangia of each inoculum type.

RESULTS

Disease Severity Assays

Field trial. Plants inoculated with sporangia from leaflets were much more severely diseased than were plants inoculated with sporangia from pure culture. All assessments were done at 144 hai. The “Newark Valley” isolate from leaves (NVL)

produced 40% disease in trial 1 compared to 15% produced by sporangia from pure culture. In trial 2 the disease severities were 30% (NVL) compared to 10% (NVP). The differences for isolate 112 were more striking. In trial 1 112L produced 50% disease, but 112P produced only 5%. In trial 2, the differences were 55% and 1%, respectively (Table 3).

Inoculum Type	Field Trial 1 Disease Severity (144hai)	Field Trial 2 Disease Severity (144hai)
Water	0 ^A	0 ^a
NVP	15 ^C	10 ^c
NVL	40 ^E	30 ^e
112P	5 ^B	1 ^b
112L	50 ^D	55 ^d

Table 3. Average disease severity (% disease) measured at 144hai for both field trials. Treatments with the same letter in superscript are not statistically different at α 0.05



Figure 2. Example tops of tomato plants cut and photographed after disease assessment at 144hai. Percent disease (of whole plant) clockwise from top left is: NV Plate 15%, NV Leaf 40%, 112 Leaf 60%, 112 Plate 1%

Moist chambers. In each trial of the moist chamber experiments, sporangia from leaves produced more severe disease than did sporangia from pure culture. For the Newark Valley isolate, disease severity induced by sporangia from leaves was 3-4 fold greater than that induced by sporangia from plates. This difference was similar to what was observed in the field. For isolate 112, the differences were even larger, with almost no disease caused by sporangia from culture but very severe disease caused by sporangia from leaflets (Fig 3, Table 4).

Inoculum Type	Lab Trial Disease Severity (144hai)
Water	0 ^A
NVP	27 ^C
NVL	80 ^D
112P	1 ^B
112L	87 ^D

Table 4. Average disease severity (%disease) measured at 144hai for the moist chamber experiment. Treatments with the same letter in superscript are not significantly different at α 0.05

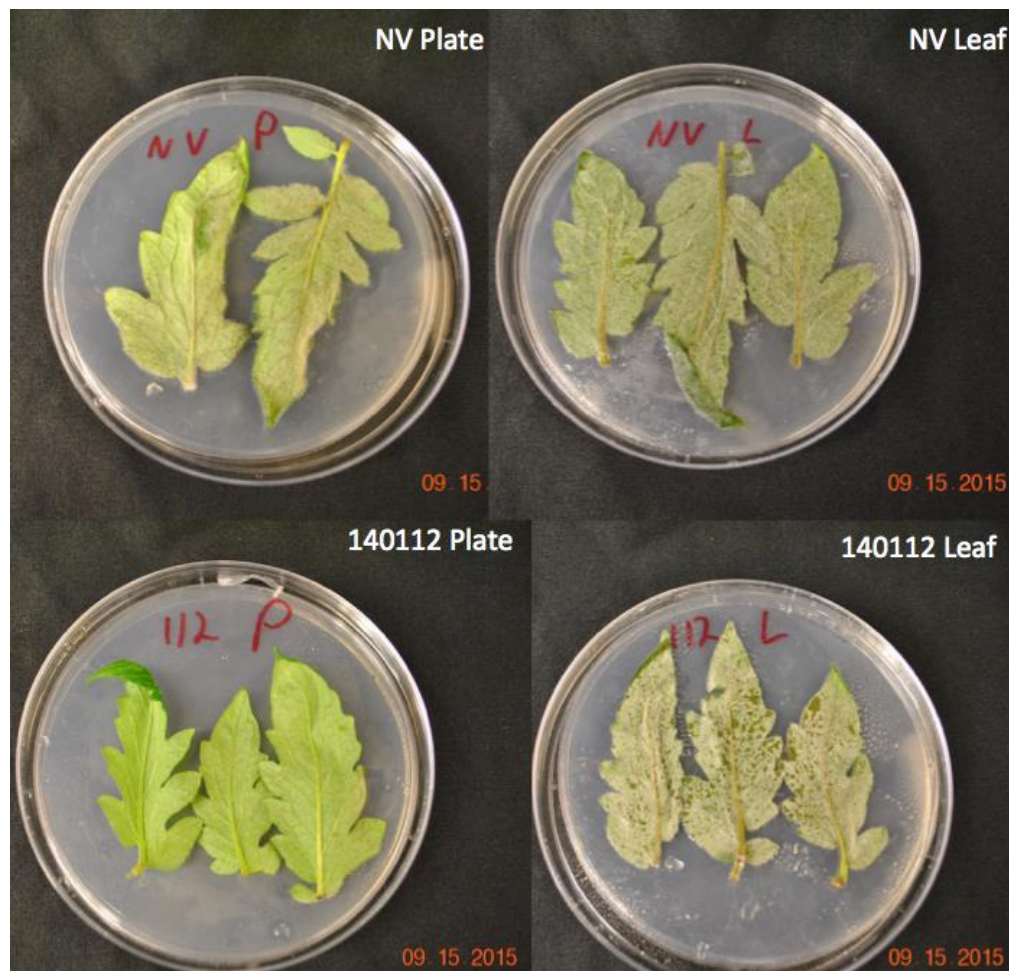


Figure 3. Example moist chambers photographed the day after disease assessment at 156hai. Percent disease (at 144hai) clockwise from top left is: NV Plate 30%, NV Leaf 90%, 112 Leaf 90%, 112 Plate 1%

Gene Expression in Sporangia

Using real-time
qPCR the expression
of two *P. infestans*

	GC P	GC L	NVP	NVL	112P	112L
INF-1	4.0	1.5	127.9	23.2	58.0	11.3
RxLR	4.7	1.8	3.7	36.3	8.7	99.2

Table 5. Gene expression of INF-1 and an RxLR from concentrated sporangia of GC, NV, and 112 from both pure culture and diseased leaflets. All expression is normalized to Actin. Data represents one of two replications, normalized expression levels were very similar between both reps.

genes, an RxLR and

an ATP binding cassette (INF-1), were compared between sporangia derived from culture plates and sporangia derived from leaflet lesions. Comparisons were conducted for all three isolates (112, NV, and GC). Sporangia derived from culture plates had higher expression of INF-1 than did sporangia derived from leaflet lesions (Table 5). There was some diversity in the expression of the RxLR effector. For the two isolates that had been passaged in pure culture on medium for more than six months (NV and 112), the expression of the RxLR effector was very much down-regulated in plate-derived sporangia compared to that in the leaflet-derived sporangia (Table 5). For these two isolates, the ratio of INF-1 change to RxLR change (Δ INF-1 : Δ RxLR) was > 50-fold (Table 5). For the isolate that had been in culture for only a few weeks (GC), expression of the two different genes was much closer and probably not significantly different (Table 5).

Because these experiments occurred 6 months after the field and moist chamber experiments, the aggressiveness of these sporangia was also assessed in detached leaflet assays (Table 6). Isolate 112, which by that time had been in culture for 24 months, was not pathogenic (Table 6). Sporangia of Isolate NV from leaflet lesions produced approximately

Isolate	% Disease (-P)	% Disease (-L)
112	0	0
NV	5	90
GC	40	90

Table 6. Re-assessment of sporangial aggressiveness after extended time in culture. Disease severity (% disease) on detached leaflets measured at 144hai

90% disease as compared to 5% disease for sporangia from pure culture. The source of the sporangia had less effect on the aggressiveness of isolate GC. (This isolate had been in culture

only a few weeks prior to this assessment.) For isolate GC, sporangia from leaflets caused twice as much disease as sporangia from culture (Table 6, Figure 4). Moist chambers of this experiment were photographed at 144hai and are shown in Figure 4.

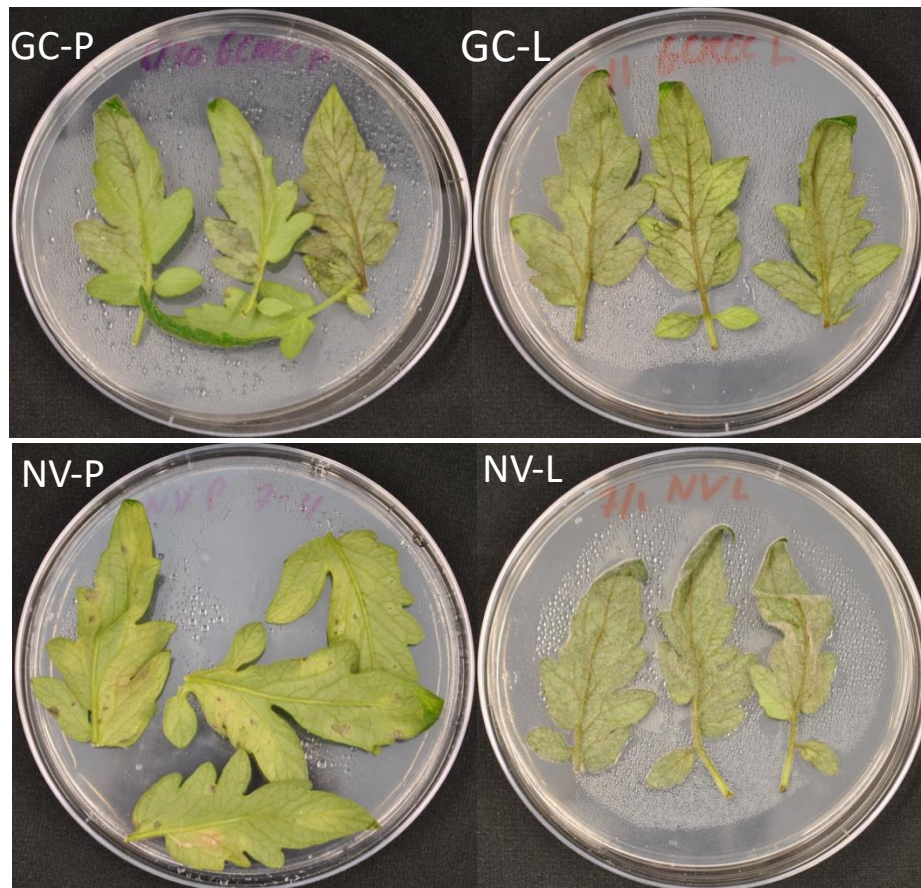


Figure 4. Disease severity on tomato leaflets inoculated with two different isolates of US-23 with different histories of culturing. Percent disease clockwise from top left: GC-P 40%, GC-L 90%, NV-L 90%, NV-P 5%.

DISCUSSION

In all experiments, sporangia derived from sporulating lesions on leaflets caused much more severe disease than did the same number of sporangia from pure cultures on agar. In field experiments, the difference ranged from 3-fold (isolate NV) to 10-fold or more (isolate 112). In the moist chamber experiments the difference ranged from 3-fold (isolate NV) to well more than 10-fold for isolate 112 (Table2). While it was not my purpose to evaluate the effect of length of

time in pure culture on aggressiveness of sporangia, isolate 112 which had been in pure culture for about one year, was dramatically less pathogenic than isolate NV which had been in pure culture for only a few passages. In the subsequent moist chamber experiment incorporating isolate GC roughly one year after initial assessment, this trend associated with culture duration held true. GC, which was then the most recently cultured isolate produced 90% disease at 144 hai from leaflet-sourced sporangia and 40% disease from culture-derived sporangia, numbers which mirror the behavior of isolate NV a year earlier (Figure 3). Then in culture for a year, isolate NV also produced 90% disease in chambers inoculated with leaflet-derived sporangia, but produced little disease from culture. These levels of disease resemble isolate 112 a year earlier, when these isolates were of similar age (Figure 3).

Assessment of gene expression in sporangia using real-time PCR revealed regulation trends associated with overall disease. Sporangia expressing more INF-1 than the RxLR resulted in little or no disease, whereas sporangia expressing equivalent or greater amounts of RxLR nearly always resulted in disease.

RxLRs are secreted effectors associated with biotrophic growth.²¹ In *P. infestans*, RXLR effector genes have been shown to be expressed predominantly during the biotrophic phase of the interaction.^{22,23} Upregulation of genes

of this type is likely important in successful infection, and this upregulation can be seen as an advantage for leaflet-derived sporangia in quickly establishing biotrophic growth. Conversely, upregulation of the elicitor INF-1 is

	GC P	GC L	NV P	NV L
INF-1	4	1.5	127.9	23.2
RxLR	4.7	1.8	3.7	36.3
Disease	40	90	5	90

Table 7. INF-1 and RxLR gene expression normalized to actin, compared to average disease at 144 hai for each inoculum type. No comparison was made for 112, as sufficient growth on plant tissue could not be established for inoculum production.

associated with late stage necrotrophic growth and growth in agar culture.²⁴ While there is no known resistance to *P. infestans* in tomato or potato as a result of INF-1 expression, host identification of INF-1 is known to trigger hypersensitive response in tobacco resulting in resistance.²⁴ It is reasonable to expect that in order for *P. infestans* sporangia expressing such comparatively high levels of detrimental elicitors like INF-1 to successfully colonize a host, significant changes in expression would need to take place. I hypothesize that the need for this change in gene expression contributes to the difference in disease severity observed in plants inoculated with pure culture-derived sporangia.

It appears that for *P. infestans*, sporangia obtained from pure culture have lower aggressiveness compared to sporangia grown on live tissue. This held true for every isolate assessed, and across each experiment type. This effect was visible in isolates passed through pure culture as few as two times from isolation, as well as in isolates that were reintroduced onto plant tissues after a year or more of culture growth.

The length of time in pure culture appears to influence pathogenicity of an isolate. After two years in culture isolate 112 was barely pathogenic at all. After one year in culture, isolate NV was reduced in pathogenicity compared to the year before; however this reduced pathogenicity could be reversed by passaging the isolate through leaflets several times. As time in culture proceeds, it appears to be increasingly difficult for an isolate to regain pathogenicity.

The phenotypic effects of this culture history on aggressiveness may be the result of changes in gene expression. Preliminary investigation of INF-1 and RxLR expression revealed that INF-1 was up regulated and RxLR was down-regulated in sporangia from culture plates as compared to those from leaflets. Subsequent analysis of diseased tissue and sporangial suspensions using RNA sequencing should provide a broader insight into changes in gene

expression associated with growth substrate. RNA sequencing may also provide some better insight into transcriptional changes associated with culture age, but more samples and more diverse ages will be necessary for a thorough examination. Time courses of less than a year apart would certainly provide better resolution in exploring long-term effects of culture growth on *P. infestans*.

The significant reduction in aggressiveness of sporangia derived from artificial medium in pure culture compared to the aggressiveness of sporangia derived from leaflet lesions has implications for many experiments done with *P. infestans*. My comparison involved only a single artificial medium, and it's logical to ask if the effect is associated only with pea/rye agar. I did not evaluate the aggressiveness of sporangia from pure cultures on other media. However, the fact that other investigators have reported using extremely high numbers of sporangia from cultures grown on a diversity of media in their inoculations^{7,9-11}, suggests that high concentrations might be necessary, and the effect that I've documented with pea/rye agar may be generally applicable to other media.

These effects may be more than quantitative. The relatively larger gene expression of INF-1 in culture-derived sporangia compared to that in lesion-derived sporangia might conceivably contribute to some qualitative differences. One such example was described in the introduction. When assessing disease progression using culture-derived sporangia, arachidonic acid seemed to have the effect of priming the plant, increasing host resistance. However, that effect was not observed using sporangia derived from leaf lesions (Fig. 1). It is possible that the combined effect of arachidonic acid and the heightened expression of INF-1 in culture-derived sporangia could inhibit growth of *P. infestans* in vivo. However, because sporangia in an epidemic will all be from leaflet lesions, one would predict that arachidonic acid would have

little to no disease suppressive effect in an agro-ecosystem.

This effect should be a significant consideration when investigating plant-pathogen interactions for *P. infestans*. Clearly, the medium used to produce sporangia for pathogenicity assays can have a significant effect. When drawing conclusions about virulence in *P. infestans* it is important to consider the broader effects of the inoculum used in such studies, and how those may influence the assessment at hand. Given these results in *P. infestans*, it would be prudent to consider inoculum source more seriously in the study of any plant-pathogen interaction, as the effects on inoculum aggressiveness conferred by culturing methods may not be restricted to *P. infestans* alone.

References

1. Stevens, N. E. *The dark ages in plant pathology in America: 1830– 1870*. **23**, 435–446 (J. Wash. Acad., 1933).
2. Fry, W. E. *et al.* The 2009 Late Blight Pandemic in the Eastern United States – Causes and Results. *Plant Dis.* **97**, 296–306 (2012).
3. Haverkort, A. J. *et al.* Societal costs of late blight in potato and prospects of durable resistance through cisgenic modification. *Potato Research* **51**, 47–57 (2008).
4. Fry, W. E. *et al.* Five Reasons to Consider *Phytophthora infestans* a Reemerging Pathogen. *Phytopathology* **105**, 966–81 (2015).
5. Fry, W. E. *Phytophthora infestans*: New Tools (and Old Ones) Lead to New Understanding and Precision Management. *Annu. Rev. Phytopathol.* **54**, 529–547 (2016).
6. Porter, L. D. & Johnson, D. A. Survival of *Phytophthora infestans* in Surface Water. *Phytopathology* **94**, 380–387 (2004).
7. Colon, L., Nielsen, B. & Darsow, U. Detached leaf test for foliage blight resistance. 14–16 (2006).
8. Larsen, M. K. G., Jørgensen, M. M., Bennike, T. B. & Stensballe, A. Time-course investigation of *Phytophthora infestans* infection of potato leaf from three cultivars by quantitative proteomics. *Data Br.* **6**, 238–248 (2016).
9. Vargas, A. M. *et al.* Characterization of *Phytophthora infestans* populations in Colombia: first report of the A2 mating type. *Phytopathology* **99**, 82–88 (2009).

10. Solano, J., Acuña, I., Chauvin, J. & Brabant, P. In-Vitro Evaluation of Resistance to Late Blight (*Phytophthora infestans* (Mont.) De Bary) in *Solanum* Accessions Native to Chile , by Inoculation of Detached Leaflets. *Am, j. Plant Sci.* **7**, 581–589 (2016).
11. Savazzini, F. & Galletti, S. Phenotypic and genotypic characterization of Italian *Phytophthora infestans* isolates. *Phytopathol. Mediterr.* **54**, 524–530 (2014).
12. Zuluaga, A. P. *et al.* Analysis of the Tomato Leaf Transcriptome During Successive Hemibiotrophic Stages of a Compatible Interaction with the Oomycete Pathogen *Phytophthora infestans*. *Mol. Plant Pathol.* **17**, 42-54 (2016).
13. Kadish, D. & Cohen, Y. Fitness of *Phytophthora infestans* isolates from metalaxyl-sensitive and -resistant populations. *Phytopathology* **78**, 912–915 (1988).
14. Mizubuti, E. S., Aylor, D. E. & Fry, W. E. Survival of *Phytophthora infestans* Sporangia Exposed to Solar Radiation. *Phytopathology* **90**, 78–84 (2000).
15. Manosalva, P. M. *et al.* Methyl Esterase 1 (*StMES1*) Is Required for Systemic Acquired Resistance in Potato. *Mol. Plant-Microbe Interact.* **23**, 1151–1163 (2010).
16. Danies, G., Small, I. M., Myers, K., Childers, R. & Fry, W. E. Phenotypic Characterization of Recent Clonal Lineages of *Phytophthora infestans* in the United States. *Plant Dis.* **97**, 873–881 (2013).
17. Jaime-Garcia, R. *et al.* Temporal and spatial patterns of genetic structure of *Phytophthora infestans* from tomato and potato in the Del Fuerte Valley. *Phytopathology* **90**, 1188–1195 (2000).

18. Caten, C. E. & Jinks, J. L. Spontaneous variability of single isolates of *Phytophthora infestans* . I. Cultural variation. *Can. J. Bot.* **46**, 329–348 (1968).
19. James, C. A Manual of Assessment Keys for Plant Diseases Canada Dep. of Agriculture. Publication No. 1458. Am. Phytopathol. Soc. (1971).
20. Pfaffl, M. Quantification strategies in real-time PCR. *A-Z Quant. PCR* 87–112 (2004).
21. Zuluaga, A. P. *et al.* Transcriptional Dynamics of *Phytophthora infestans* During Sequential Stages of Hemibiotrophic Infection of Tomato. *Mol. Plant Pathol.* **17**, 29-41 (2016).
22. van West, P., de Jong, A J., Judelson, H. S., Emons, A M. & Govers, F. The *ipiO* gene of *Phytophthora infestans* is highly expressed in invading hyphae during infection. *Fungal Genet. Biol.* **23**, 126–138 (1998).
23. Kelley, B. S. *et al.* A secreted effector protein (SNE1) from *Phytophthora infestans* is a broadly acting suppressor of programmed cell death. *Plant J.* **62**, 357–366 (2010).
24. Kamoun, S. *et al.* A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. *Mol Plant Microbe Interact* **10**, 13–20. (1997).